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# The effect of inhaled nitric oxide treatment on biomarkers of oxidative/nitrosative damage to proteins and DNA/RNA

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ABSTRACT

Inhaled nitric oxide (iNO) is a selective pulmonary vasodilator that is used as a treatment for persistent pulmonary hypertension in neonates (PPHN) with hypoxic respiratory failure. The generation of reactive oxygen and nitrogen species might induce oxidative/nitrosative damage to multiple organs. There is an increasing scientific and clinical interest in the determination of specific biomarkers to measure the degree of oxidative/nitrosative stress in non-invasively collected biofluids. A method for the simultaneous detection of a panel of oxidative and nitrosative stress-related biomarkers for quantifying damage to proteins and DNA/RNA in 20 μL of infant urine samples based on reversed-phase ultra-performance liquid chromatography coupled to tandem mass spectrometry operating in positive electrospray ionization mode ($ESI^+$) was optimized and validated. Infant urine samples from two different studies were analyzed: (i) term and preterm infants from a nutrition study (Nutrishield, N = 50) and (ii) infants with respiratory insufficiency, including infants with PPHN (N = 16) that required iNO treatment and a control group without treatment (N = 14). Eleven of 14 metabolites were detected in >50 % of infant urine samples, with ranges between 0.008 and 1400 μmol/g creatinine. When comparing across groups, differences in samples collected after iNO treatment in comparison to the rest of the groups were found for m-tyrosine (*m*-Tyr and *m*-Tyr/Phe) and ortho-tyrosine (*o*-Tyr and *o*-Tyr/Phe) (*p*-values <0.001, Wilcoxon rank-sum test). Positive linear relationships were found with NO exposure corrected by infant weight for *m*-Tyr, *m*-Tyr/Phe, *o*-Tyr, *o*-Tyr/Phe and 3-nitrotyrosine. Future studies will focus on the evaluation of the impact of iNO treatment on health and oxidative/nitrosative stress-related morbidities associated with prematurity.

## 1. Introduction

Persistent pulmonary hypertension (PPHN) is a critical medical condition affecting the newborn infant and characterized by elevated pulmonary vascular resistance and reduced pulmonary blood flow that causes profound hypoxemia and may have deleterious consequences for

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the neonate. Thus, ensuring both an early diagnosis and treatment is crucial for reducing morbidity and/or mortality. One of the key therapeutic interventions for PPHN is inhaled nitric oxide (iNO), a selective pulmonary vasodilator that has drastically improved the management of this condition [1]. As a selective pulmonary vasodilator, iNO improves oxygenation and reduces the need for more invasive interventions, such as extracorporeal membrane oxygenation (ECMO). Moreover, iNO has also been recently employed for postnatal stabilization [2,3]. In a randomized placebo-controlled study, 20 ppm of iNO was used to avoid the use of higher oxygen concentrations during preterm infant stabilization in the delivery room. iNO was tapered at the end of the resuscitation period, and although both groups showed similar clinical outcomes, the intervention group required less oxygen for stabilization [3]. However, while iNO can be highly effective, its use should be carefully monitored due to its potential toxicity, particularly in prolonged or high-dose regimens [4]. Of note, in a recent epidemiological study the use of iNO in preterm infants during their stay at the neonatal intensive care unit was independently associated with an increased risk of childhood cancer [5]. These studies have sparked scientific interest in the determination of specific biomarkers in non-invasively collected biofluids for short- and long-term follow-up of the safety of iNO therapy [6].

In the presence of endogenous nitric oxide (NO) soluble guanylate cyclase (sGC) is activated to produce the secondary messenger guanosine-3′,5′-cyclic monophosphate (cGMP), that activates protein kinases (PKGs). Through the production of cGMP, sGC can exert many physiological effects mediating vascular smooth and phototransduction [7]. Nevertheless, NO is a free radical that in combination with anion superoxide ($O_2^{\bullet-}$), produces peroxynitrite ($ONOO^-$), a highly reactive nitrogen species (RNS), responsible for various critical biological processes, including protein nitration and nitrosylation which can lead to nitrosative stress and precede oxidative stress [8]. The main RNS are $ONOO^-$ and its reduction product nitrogen dioxide ($^{\bullet}NO_2$) [9], which is a potent toxic oxidant that irreversibly produces peroxidised lipids and carbonylated proteins, causing cell death [10]. Oxidative/nitrosative stress represents a redox imbalance and plays a role in the deterioration of organs and systems, currently recognized to be involved in cardiometabolic pathologies [10], inflammatory responses [11], neurodegenerative diseases [12], and cancer [13].

In the presence of oxidative stress, proteins and DNA/RNA become vulnerable to oxidation due to the interaction with reactive oxygen species (ROS) such as hydroxyl radical ($^{\bullet}OH$) or superoxide anion ($O_2^{\bullet-}$) [14]. The most-studied oxidative catabolites of DNA and RNA used as biomarkers are 8-hydroxy deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHGuo), respectively [15]. On the other hand, biomarkers reflecting damage to proteins by ROS have been previously reported in infant urine [16], including meta-tyrosine (*m*-Tyr) and ortho-tyrosine (*o*-Tyr), which derive from free radical oxidation of phenylalanine (Phe). Additionally, RNS can also modify nucleic acids forming 8-nitroguanine (8-$NO_2$-Gua) [17,18] and 8-nitroguanosine (8-$NO_2$-Guo) [19]. Furthermore, 3-nitrotyrosine (3$NO_2$-Tyr) and 3-chlorotyrosine (3Cl-Tyr) both originated from the oxidation of para-tyrosine (p-Tyr), are induced by peroxynitrite ($ONOO^-$) and hypochlorous acid ($HClO^-$), respectively [16,20,21]. Both compounds serve as biomarkers for nitrosative stress and inflammation, respectively, as well as indicators of myeloperoxidase (MPO) activity [22].

The available approaches to analyze the oxidative/nitrosative stress biomarkers have increased in number and diversity. There have been reported analytical techniques such as fluorometry [23], immunoassay [24], and colorimetric commercial kits [25]. However, many literature reports are obviating methodological details and limitations. Due to the selectivity and sensitivity provided by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with soft ionization sources (i.e., electrospray ionization, ESI), this technique is considered the gold standard for the detection of oxidative/nitrosative stress biomarkers in a wide range of biofluids such as cerebrospinal fluid [26–28], human milk [14,29], amniotic fluid [30,31], plasma [32], and urine [16,32,33]. The use of non-invasive biofluids, such as urine, provides a practical and ethical method to monitor these biomarkers in neonates, however, data on infant urine undergoing iNO therapy remain scarce. The evaluation of oxidative and nitrosative stress biomarkers in neonates with PPHN undergoing iNO treatment provides crucial insights into the therapy's molecular effects, enabling a better understanding of its potential risks and guiding safer clinical applications.

This work aimed at the quantification of a comprehensive panel of biomarkers of oxidative and nitrosative damage to DNA, RNA, and proteins in infant urine and their relevance in the context of iNO treatment of the newborn. Although the efficiency of iNO as a therapeutic agent for PPHN is confirmed, its short and long-term toxicity remains unknown. ROS/RNS might induce oxidative/nitrosative damage to multiple organs and activate relevant metabolic, inflammatory, and pro-apoptotic pathways. The detection of specific biomarkers to measure the degree of oxidative/nitrosative stress may provide meaningful insight into the pathophysiology of NO-derived clinical conditions. The specific objectives of this study were to: (1) quantify oxidative and nitrosative stress biomarkers in neonates undergoing iNO therapy; (2) compare these biomarkers across different clinical contexts, including untreated neonates and healthy controls; and (3) explore potential correlations between iNO exposure and biomarker levels to identify dose- and time-dependent effects. To the best of our knowledge, this is the first time that oxidative and nitrosative stress biomarker concentrations in infant urine from patients with PPHN undergoing iNO treatment have been reported. These findings will serve as a foundation for future large-scale studies to elucidate the broader clinical implications of oxidative and nitrosative stress in this population.

## 2. Material and methods

### 2.1. Standards and reagents

Ultrapure water was generated using a Milli-Q Integral Water Purification System from Merck Millipore (Darmstadt, Germany). Formic acid 98 % (FA) was obtained from Panreac (Barcelona, Spain). Pure analytical standards of phenylalanine (Phe), para-tyrosine (*p*-Tyr), nucleoside 2′-deoxiguanosine (2 dG), ortho-tyrosine (*o*-Tyr), meta-tyrosine (*m*-Tyr), 3-chlorotyrosine (3-Cl-Tyr), 3-nitrotyrosine (*$NO_2$*-Tyr), 8-oxo-2′-deoxyguanosine (8OHdG), 2′-deoxyguanosine (2-dG), guanine (Gua), and guanosine (Guo) were obtained from Sigma-Aldrich (St. Louis, MO, USA) (purities >96 % w/w). Guanosine-3′,5′-cyclic monophosphate (cGMP) sodium salt (>98 %), 8-hydroxyguanosine (8OHGuo), 8-nitroguanine (8$NO_2$-Gua) (>95 %), and 8-nitroguanosine (8$NO_2$-Guo) were acquired from Biolog Life Science Institute (Flughafendamm 9a, Bremen, Germany) with purities >95 % w/w.

The internal standard (IS) *p*-Tyr-$D_2$ (98 %) was purchased from Cambridge Isotope Laboratories, 2 dG-$^{13}C^{15}N_2$ and 8-OHdG-$^{13}C^{15}N_2$ were obtained from Santa Cruz Biotechnology (purities >98 % w/w), and Phe-$D_5$ from CDN Isotopes (Pointe-Claire, Canada), while 8-azidoadenosine (8-$N_3$-Ado) (>95 %) was obtained from Biolog Life Science Institute (Flughafendamm 9a, Bremen, Germany).

### 2.2. Infant urine samples

Urine samples were obtained within the framework of the Nutrishield study (https://nutrishield-project.eu/, https://register.clinicaltrials.gov, ID: NCT05646940) [34] and the iNOx study registered in the *Registro Español de Estudios Clínicos* (https://reec.aemps.es/reec/public/eo_detail.html#, ID: 0044-2021-OBS). These are prospective, observational cohort studies carried out at the Division of Neonatology of the University and Polytechnic Hospital La Fe (HUiP La Fe, Valencia), General University Hospital Alicante (Alicante), and Hospital Sant Joan De Déu (Barcelona). The Nutrishield study focuses on early nutrition, while the iNOx study explores and quantifies the biochemical impact of the use of iNO on newborn infants receiving this

treatment in comparison to a control group that does not receive iNO. Studies were conducted following relevant guidelines and regulations including the Declaration of Helsinki. The Ethics Committee for Biomedical Research of the Health Research Institute La Fe (Valencia, Spain) approved the study protocols (approval numbers 2019-289-1 and 507, July 21, 2021), and parents or legal representatives gave written consent to participate. Confidentiality of subjects was maintained during the study. All participants were assigned a code and data allowing personal identification will not be shared at any time. Participants may withdraw consent for participating in the study at any time.

### 2.3. LC-MS/MS analysis of nitrated amino acids and nucleotides in infant urine

The sample preparation workflow is summarized in Fig. 1A. Briefly, for infant urine collection, sterile cotton pads were placed in the diaper [34]. Cotton pads were collected after 1 h and squeezed with a sterile polypropylene syringe. Urine samples were aliquoted to avoid freeze-thaw cycles and stored at −80 °C until further analysis. Urine samples were defrosted on ice and homogenized on a Vortex® mixer. Samples were centrifuged at 12.000×*g*, 4 °C, 10 min for protein elimination. Two aliquots of 10 μL of urine supernatant each were collected. One aliquot was diluted 1:10 and another 1:100 with 0.1 % v/v FA and an internal standard mixture containing *p*-Tyr-$D_2$, 2 dG-$^{13}C^{15}N_2$, 8OHdG-$^{13}C^{15}N_2$ and Phe-$D_5$ at a final concentration of 1 μM and 8-$N_3$-Ado at 0.02 μM.

An Acquity-Xevo TQS system from Waters (Milford, MA, USA) using positive electrospray ionization ($ESI^+$) mode was employed for UPLC-MS/MS analysis. Chromatographic separation and ESI interface conditions were optimized using pure analytical standard solutions of individual analytes (1 μM) or selected according to previously published methods with some modifications [16]. Separations were performed on a Waters Acquity UPLC HSS T3 (100 × 2.1 mm, 1.8 μm) column using $H_2O$ 0.1 % v/v formic acid (channel A) and $CH_3CN$ (0.1 % formic acid v/v) (channel B) binary gradient. Flow rate, column temperature and injection volume were set at 0.3 mL $min^{-1}$, 50 °C, and 5 μL, respectively. The gradient with a total run time of 7.0 min was as follows: from 0 to 2 min, 3 % v/v of channel B; from 2 to 4.5 min %B increased up to 95 %; from 4.5 to 5.8 min conditions were held constant at 95 % B followed by the return to initial conditions (i.e., 5 % B) between 5.8 and 6.0 min; conditions were maintained for 1 min for system re-equilibration. For MS data acquisition, the following instrumental conditions were selected: source and desolvation temperatures were set to 150 and 600 °C, respectively; nitrogen cone and desolvation gas flows were set to 150 and 1000 L $h^{-1}$, respectively. Dwell time was selected to ensure a minimum of 10 data points per peak. MS detection was carried out by multiple reaction monitoring (MRM) employing the acquisition parameters summarized in Table 1.

### 2.4. Method validation

The validation of the method followed the guidelines for bioanalytical method validation by the US Food and Drug Administration (FDA) [35]. A range of figures of merit were evaluated in three independent experiments, including linear range, precision, accuracy, selectivity, limit of detection (LOD), lower limit of quantification (LLOQ), and carry-over. The accuracy was calculated as the relative standard deviation (%RSD) of replicate standards within one validation batch (intra-day) and between validation batches (inter-day). The LLOQs were established as the minimum concentration of analyte that can be measured with an imprecision of less than 20 %, and LODs were estimated as a ratio of 3/10 x LLOQ. The acceptance criteria were as follows: calibration curves should include at least 6 points excluding the blank, the %RSD of standard and spiked samples replicates (n = 3) should be below 20 % at low concentration and below 15 % at mid and high concentrations, and coefficients of determination ($R^2$) should be higher than 0.990. Concentration ranges were carefully chosen based on expected values and inter- and intra-individual variability to ensure accurate and precise quantification of metabolites in urine samples. Three different levels were stated for spiked urine samples at depending on the compound concentration found in the sample. Calibration curves were constructed using a zero calibrator (i.e., blank with IS) and ten standard solutions covering the selected concentration ranges.

### 2.5. Software, statistical analysis, and data availability

LC–MS data were acquired and processed using MassLynx™4.1 from Waters (Milford, MA, USA). Data analysis was carried out in MATLAB R2022a (MathWorks, Natick, MA, USA) and using the PLS Toolbox 9.21 (Eigenvector Research Inc., Manson, WA, USA). Continuous variables were expressed as mean ± standard deviation, median (interquartile) or mean (confidence interval, 95 %) depending on underlying data distribution. Kruskal-Wallis test was used for inter-group comparisons, and Wilcoxon rank-sum test ($\alpha = 0.05$) was used for pairwise comparisons between two groups. Additionally, Wilcoxon rank-sum test ($\alpha = 0.05$) was also employed for concentration comparison between biomarker levels in sample groups. The Pearson's correlation coefficient was used for assessing paired associations among metabolite concentrations. Principal Component Analysis (PCA) was carried out using autoscaled data normalized to creatinine and biomarker ratios (normalized with their respective precursors). Data obtained is shared in Supplementary Table 1.

## 3. Results and discussion

### 3.1. Characteristics of study population

A total of 94 urine samples from 80 infants were collected, and categorized into three different groups: i) infants enrolled in the reference group (Nutrishield study), including samples from preterm infants (<32 weeks of gestation) collected when achieving complete enteral nutrition and term infants when recovering birth weight (N = 50 including 25 pre-term and 25 term infants); and infants enrolled in the iNOx study ii) with PPHN receiving iNO treatment (N = 16) and iii) without PPHN and iNO treatment (control group, N = 14). From the group receiving iNO treatment, samples were collected before initiation of iNO treatment (T0, N = 5), after a minimum exposure of 1 h to iNO (T1, N = 14), and 6 h after withdrawal of iNO treatment (T2, N = 11). Demographic, clinical, and perinatal characteristics of the participants were recorded in Table 2.

The use of NO has been associated with elevated levels of methemoglobin (metHb), and high doses of NO have been observed to result in increased metHb [36,37]. In our study group, all patients presented metHb values < 5 %. In infants receiving treatment with iNO, $NO_2$ was continuously measured, and values were <0.5 ppm in all cases.

Significant differences were observed between groups for Apgar score at 1 and 5 min with ($p < 0.05$) and ($p < 0.001$), respectively. Additionally, significant differences were found for postnatal age, intubation and $FiO_2$ in the delivery room, $PaO_2/FiO_2$ ratio, positive pressure ventilation, and inhaled nitric oxide (iNO) treatment (all *p*-values <0.001). SNAPPE-II scores also showed a significant difference between groups ($p < 0.05$). These results reflect heterogeneity in the pathophysiological conditions among the studied groups of infants. The Nutrishield reference group represents a scenario where infants are overall in better conditions as compared to the iNOx groups. The cases group consisted of infants that presented ecographic or clinical signs of PPHN, and the clinical outcomes were remarkably distinct from the iNOx control group without PPHN.

### 3.2. Analytical method validation

Chromatographic conditions were optimized employing a working

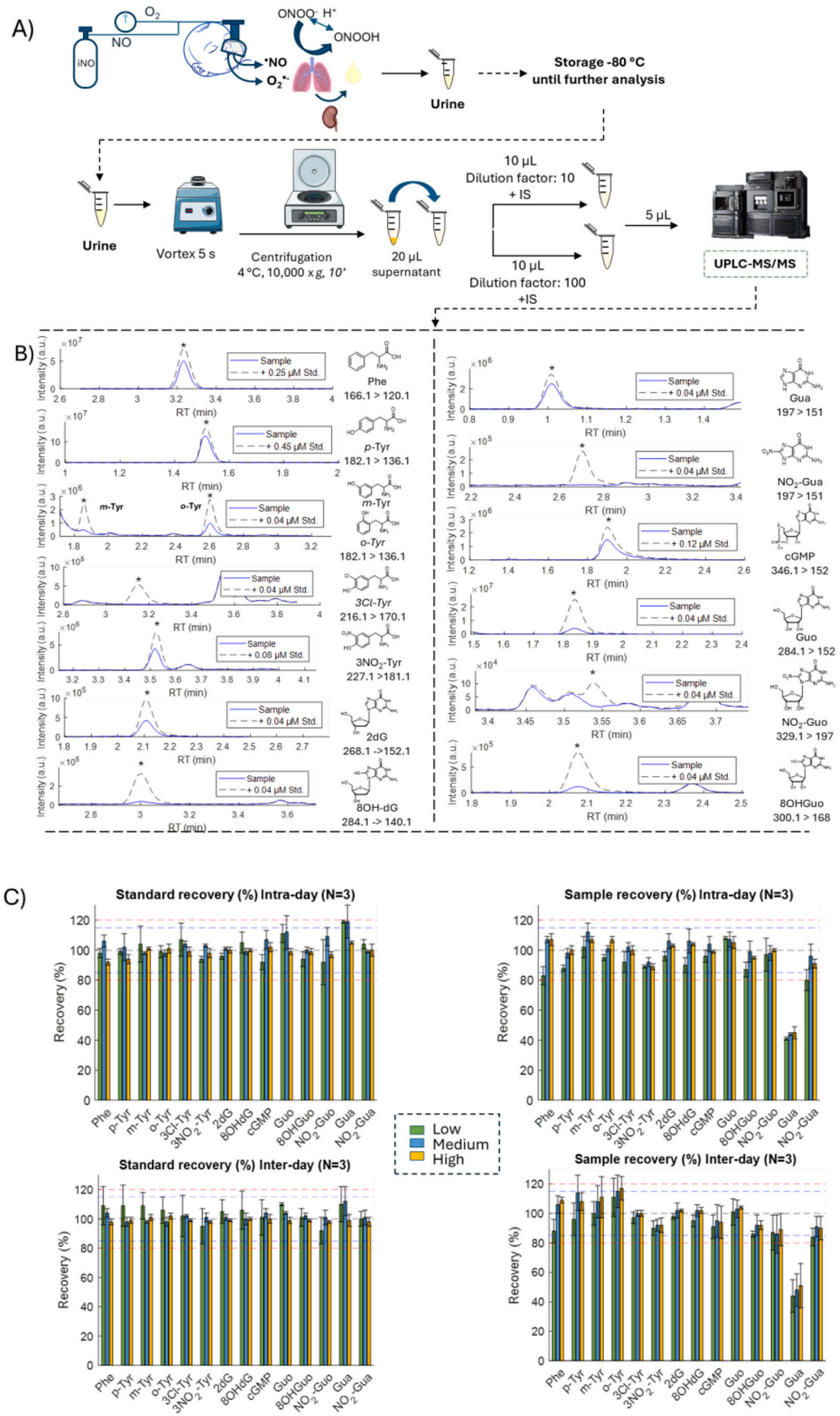


**Fig. 1.** Analytical workflow, method validation and LC-MS/MS profiles of detected and quantified compounds in infant urine. A) Schematic illustration depicting the impact of iNO and $O_2$ therapy in neonates, sample collection, processing, and analysis. B) Chromatographic traces of detected compounds. C) Results from method validation employing a spiked sample.

**Table 1**
Acquisition parameters and main figures of merit of the LC-MS/MS method.

| Oxidative/ Nitrosative damage | Analyte | *m/z* Parent ion | Cone [V] | CE [eV] | *m/z* Daughter ion | RT ± s (min) | | Spiking concentration (L/M/H) [nM] | Calibration range (nM) | | $R^2$ | LOD (nM) | LLOQ (nM) | LLOQ (nM) (in urine) | IS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Protein oxidation/ nitration** | *o*-Tyr | 182.1 | 20 | 10 | 136.1 | 2.65 | ± 0.019 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | *p*-Tyr-$D_2$ |
| | *m*-Tyr | 182.1 | 20 | 10 | 136.1 | 1.89 | ± 0.016 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | *p*-Tyr-$D_2$ |
| | *p*-Tyr | 182.1 | 20 | 10 | 136.1 | 1.54 | ± 0.010 | 225/450/900 | 7 | – 2850 | 0.998 | 2 | 7 | 700 | *p*-Tyr-$D_2$ |
| | 3Cl-Tyr | 216.1 | 30 | 15 | 170.1 | 3.23 | ± 0.030 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | Phe-$D_5$ |
| | 3$NO_2$-Tyr | 227.1 | 25 | 10 | 181.1 | 3.55 | ± 0.006 | 40/80/160 | 0.6 | – 228 | 0.998 | 0.2 | 0.6 | 6 | 8-$N_3$-Ado |
| | Phe | 166.1 | 20 | 20 | 120.1 | 3.29 | ± 0.022 | 125/250/500 | 7 | – 2850 | 0.998 | 2 | 7 | 700 | Phe-$D_5$ |
| | Phe-$D_5$ | 171.1 | 20 | 20 | 125.1 | 3.21 | ± 0.017 | – | – | – | – | – | – | – | – |
| | *p*-Tyr-$D_2$ | 184.1 | 15 | 20 | 138.1 | 1.53 | ± 0.011 | – | – | – | – | – | – | – | – |
| **DNA & RNA oxidation/ nitration** | 2 dG | 268.1 | 25 | 15 | 152.1 | 2.20 | ± 0.016 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | 2dG-$^{13}C^{15}N_2$ |
| | 8OHdG | 284.1 | 30 | 15 | 140.1 | 3.15 | ± 0.029 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | 8OHdG-$^{13}C^{15}N_2$ |
| | Guo | 284.1 | 10 | 15 | 152 | 1.91 | ± 0.013 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | *p*-Tyr-$D_2$ |
| | 8OHGuo | 300.1 | 25 | 15 | 168 | 2.16 | ± 0.017 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | 8OHdG-$^{13}C^{15}N_2$ |
| | $NO_2$-Guo | 329.1 | 10 | 10 | 197 | 3.56 | ± 0.006 | 20/40/80 | 0.3 | – 114 | 0.998 | 0.09 | 0.3 | 3 | *p*-Tyr-$D_2$ |
| | Gua | 152.1 | 20 | 20 | 135 | 1.02 | ± 0.006 | 20/40/80 | 0.3 | – 114 | 0.996 | – | – | 3 | *p*-Tyr-$D_2$ |
| | $NO_2$-Gua | 197.0 | 15 | 20 | 151 | 2.81 | ± 0.020 | 20/40/80 | 0.3 | – 114 | 0.997 | 0.09 | 0.3 | 3 | 8OHdG-$^{13}C^{15}N_2$ |
| | cGMP | 346.1 | 35 | 15 | 152 | 1.98 | ± 0.022 | 60/120/240 | 1.0 | – 356 | 0.998 | 0.3 | 1.0 | 10 | 8OHdG-$^{13}C^{15}N_2$ |
| | 8-$N_3$-Ado | 309.1 | 30 | 10 | 177 | 3.57 | ± 0.005 | – | – | – | – | – | – | – | – |
| | 8OHdG-$^{13}C^{15}N_2$ | 287.0 | 25 | 10 | 171 | 3.16 | ± 0.029 | – | – | – | – | – | – | – | – |
| | 2dG-$^{13}C^{15}N_2$ | 271.0 | 15 | 10 | 155 | 2.20 | ± 0.016 | – | – | – | – | – | – | – | – |

CE: Collision Energy, LOD: Limit of Detection, LLOQ: Lower Limit of Quantification. LLOQ in sample was defined considering the preconcentration factor achieved during sample processing.

**Table 2**
Characteristics of the study population.

| Parameter | Nutrishield group (N = 50) | iNOx Controls (N = 14) | iNOx Cases (N = 16) | *p*-value |
|---|---|---|---|---|
| Gestational age (weeks), median (IQR) | 37 (12) | 35 (6) | 32 (10) | NS |
| Infant birth weight (g), mean (SD) | 2204 (1083) | 2203 (1275) | 2333 (1258) | NS |
| Type of delivery (vaginal), N (%) | 26 (52) | 4 (29) | 6 (38) | NS |
| Postnatal age (days), mean (SD) | 14 (5) | 15 (12) | 4 (4) | <0.001 |
| Sex male, N (%) | 26 (52) | 7 (50) | 7 (44) | NS |
| Apgar 1 min, mean (95 % CI) | 8 (0.5) | 7 (1.1) | 6 (1.1) | <0.05 |
| Apgar 5 min, mean (95 % CI) | 9 (0.2) | 9 (0.8) | 8 (1.1) | <0.001 |
| Intubation (Delivery room), N (%) | 0 (0) | 3 (21) | 5 (31) | <0.001 |
| $FiO_2$ (Delivery room), mean (95 % CI) | – | 0.5 (0.13) | 0.8 (0.15) | <0.001 |
| PaO2/FiO2 (mmHg), mean (95 % CI) | – | 410 (40) | 110 (20) | <0.001 |
| $FiO_2$ (iNO/T0), mean (95 % CI) | 0.22 (0.02 | 0.25 (0.03) | 0.91 (0.08) | <0.001 |
| $FiO_2$ (iNO/T1), mean (95 % CI) | – | – | 0.78 (0.11) | <0.001 |
| Ventilatory support (invasive ventilation/ non-Invasive ventilation/ spontaneous), N (%) | 0/13/37 | 7/4/3 | 16/0/0 | <0.001 |
| SNAPPE-II, mean (95 % CI) | – | 14 (5) | 35 (12) | <0.05 |
| iNO treatment | | | | |
| · Max dose (ppm), mean (SD) | 0 (0) | 0 (0) | 13 (6) | <0.001 |
| · Time of exposure (hours), mean (SD) | 0 (0) | 0 (0) | 40 (30) | <0.001 |
| Mortality N (%) | – | 1 (7) | 4 (25) | NS |
| Morbidity | | | | |
| · BPD, N (%) | – | 2 (14) | 1 (6) | NS |
| · ROP, N (%) | – | 3 (21) | 2 (13) | NS |
| · NEC, N (%) | – | 1 (7) | 0 (0) | NS |

Note: BPD = Bronchopulmonary dysplasia; ROP = Retinopathy of prematurity; NEC = Necrotizing enterocolitis; SD = standard deviation; CI = confidence interval; NS = Not significant.

solution. The main characteristics and figures of merit obtained from the calibration curves including the selected MS/MS acquisition parameters and concentration ranges of employed standards are summarized in Table 1. Retention time was stable during the whole validation study with an intra-day standard deviation of ≤0.04 min. Linearity of response, as shown in Table 1, covered up to three orders of magnitude with LODs and LLOQs in the 0.09–2.1 and 0.3–7 nM range, respectively. Fig. 1B shows representative LC-MS/MS chromatograms extracted from the analysis of a urine sample and spiked urine sample with adequate peak shapes, selectivity, and resolution. Target analytes including *p*-Tyr, Phe, 8-OHdG, and 2 dG were quantified by using analogous IS. Other analytes (i.e., *o*-Tyr, *m*-Tyr, 3-Cl-Tyr, 3-$NO_2$-Tyr, Guo, 8-$NO_2$-Gua, 8-$NO_2$-Guo, 8OHGuo and cGMP) were quantified based on a surrogate IS (see Table 1). Appropriate accuracies with recoveries between 90 - 112 % and 80–117 % were observed in standard solutions and spiked urine samples, respectively, except for Gua with recovery values < 60 % at low, medium, and high levels. Precisions were ranging between 1 and 14 %RSD, as shown in Fig. 1C. In summary, the proposed method involving isotopically labelled ISs allowed to compensate matrix effects and provided adequate analytical accuracy and precision for the quantification of all compounds, evidencing an adequate method performance for the protein and DNA/RNA oxidation and nitration in urine samples except for Gua, where results obtained were semiquantitative.

### 3.3. Levels of protein and DNA/RNA biomarker in newborn's urine samples

Among the panel of 14 distinct compounds, eleven target analytes were detected in >50 % of urine samples. The frequency of detection and quantification of the different analytes are shown in Fig. 2A. 8-$NO_2$-Gua was not detected in any of the selected study samples and 8-$NO_2$-Guo was detected in only 1 % of samples. Only those compounds detected in >50 % of samples were selected for statistical analysis. In Fig. 2B an overview of the concentrations of compounds determined in urine samples after normalization by creatinine using the validated UPLC-MS/MS method is shown. The highest levels were observed for Phe and *p*-Tyr with concentrations ranging in the mmol/g creatinine range followed by cGMP, in terms of abundance. Overall, six orders of magnitude were covered for concentration levels in the analysis, with ranges between µmol/g creatinine. Additional to creatinine normalization, compound ratios with their respective precursors (i.e., o-Tyr/Phe, m-Tyr/Phe, $NO_2$-Tyr/p-Tyr, 8OH-Guo/Guo, and 8OHdG/2 dG) were evaluated, when possible.

Pearson's linear regression analysis between all compound concentrations after normalization to creatinine and ratios found in urine samples were calculated (see Fig. 2C). Both compounds normalized with creatinine and precursor ratios were selected as biomarkers for statistical analysis. Interestingly, mainly positive correlations were observed. Biomarkers of oxidative damage to proteins; *m*-Tyr with *o*-Tyr, and *o*-Tyr/Phe with *m*-Tyr/Phe presented a strong correlation among each other ($\rho > 0.90$, *p*-value <0.001). 3$NO_2$-Tyr/p-Tyr presented a positive correlation with o-Tyr/Phe and m-Tyr/Phe ($\rho > 0.2$, *p*-value <0.05), which suggests a relationship between nitrosative and oxidative stress in those patients that were exposed to NO in combination with oxygen. Regarding to the correlation between DNA and RNA biomarkers, 8OHdG/2 dG and 8OHGuo/Guo showed a positive strong correlation ($\rho = 0.4$, *p*-value <0.001). In addition, 8OHdG showed a positive correlation with, *o*-Tyr, 3$NO_2$-Tyr, 8OHGuo and cGMP. A simple observation of these results reveals that damage to DNA/RNA and proteins has not occurred in the same manner, due to different dynamic processes in molecular and cell biology [38]. In addition, cGMP presented a significant positive correlation with *m*-Tyr, *o*-Tyr, 3$NO_2$-Tyr and 8OHdG. cGMP is formed in the presence of NO, and NO/cGMP signaling plays a role in the regulation of physiological processes such as smooth muscle relaxation [2]. During iNO exposure, both ROS and RNS are present, and they might lead to the activation of NO signaling and protein/RNA/DNA oxidation simultaneously. Ratios and normalized creatinine biomarkers were significantly correlated, suggesting complementary use of both for statistical analysis and oxidative/nitrosative stress assessment.

### 3.4. Assessment of oxidative/nitrosative stress biomarker profiles in infant urine

For an overview of the distribution and variability of biomarkers in

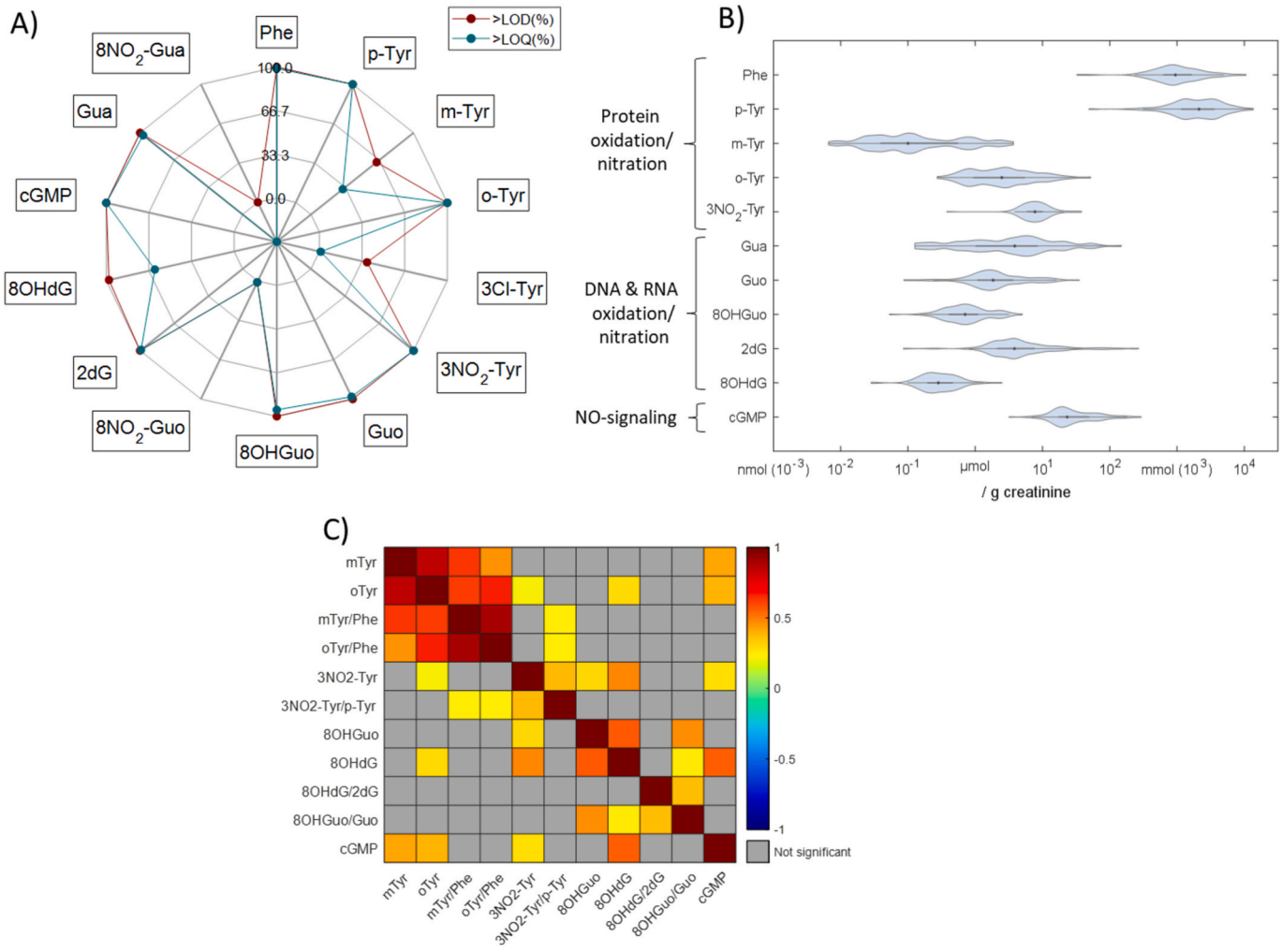


**Fig. 2.** Overview of the results from method validation and analysis of the selected biomarker panel. A) Proportion of detected and quantified compounds in infant urine samples. B) Compound concentrations found in infant urine samples. C) Pearson correlations among detected compounds and ratios.

samples depending on the patient's conditions, PCA was performed (see Supplementary Fig. 1). As shown in the scores plot (Supplementary Fig. 1, left), the samples from the Nutrishield study group were more homogeneous in comparison to high inter- and intra-individual differences observed in the iNOx groups. This observation agrees with the different characteristics of the patients included in both studies (see Table 2). While Nutrishield focused on healthy term infants and preterm infants in good general conditions to study the impact on nutrition at different gestational ages, infants recruited for the iNOx study were in worse and showed signs of oxidative and/or nitrosative stress.

The iNOx samples tended towards higher concentrations of all detected biomarkers in comparison to samples from the Nutrishield study, with the samples collected after iNO treatment (T2) showing the highest levels (see Supplementary Fig. 1, right). Specifically, biomarkers of oxidative damage to proteins (i.e., *m*-Tyr and *o*-Tyr and *m*-Tyr/Phe and *o*-Tyr/Phe) were detected at elevated concentrations at T2 (see Fig. 3-A, B, C and D). Statistically significant differences were observed between iNOx-T2 and all other sub-groups of urine samples for *m*-Tyr (Fig. 3A) and *m*-Tyr/Phe (Fig. 3B), *o*-Tyr (Fig. 3C), and *o*-Tyr/Phe (Fig. 3D). An increasing tendency was observed for *m*-Tyr and *m*-Tyr/Phe in the iNO group between samples collected from T0 to T2. Several studies have reported *m*-Tyr and *o*-Tyr as oxidative stress biomarkers, and their association with toxicity adversely affects cells and tissues, inflammation, and might potentially contribute to the development of cancer [39–41].

In Fig. 3E, 8OHGuo showed significant differences between Nutrishield and iNOx-T2 suggesting that damage to RNA is higher at T2. Interestingly, higher levels were observed in the iNOx control group for 8OHGuo/Guo (Fig. 3F), 8OHdG (Fig. 3G) as well as 8OHdG/2 dG (Fig. 3H) compared with Nutrishield and/or iNOx groups. For cGMP (Fig. 3I) a similar trend was observed, which showed significantly lower concentrations in Nutrishield samples as compared to the different iNOx subgroups. These results suggest that biomarker levels are affected not only by oxidative and nitrosative stress resulting from iNO therapy but also by baseline variations among study groups. This is further demonstrated by the elevated levels of certain biomarkers in the iNO control group. Additionally, no statistically significant differences were found for $3NO_2$-Tyr and $3NO_2$-Tyr/p-Tyr between study groups, which is in agreement with the results reported by Ballard et al. [42].

A dose-dependent elevation in cGMP levels upon iNO administration

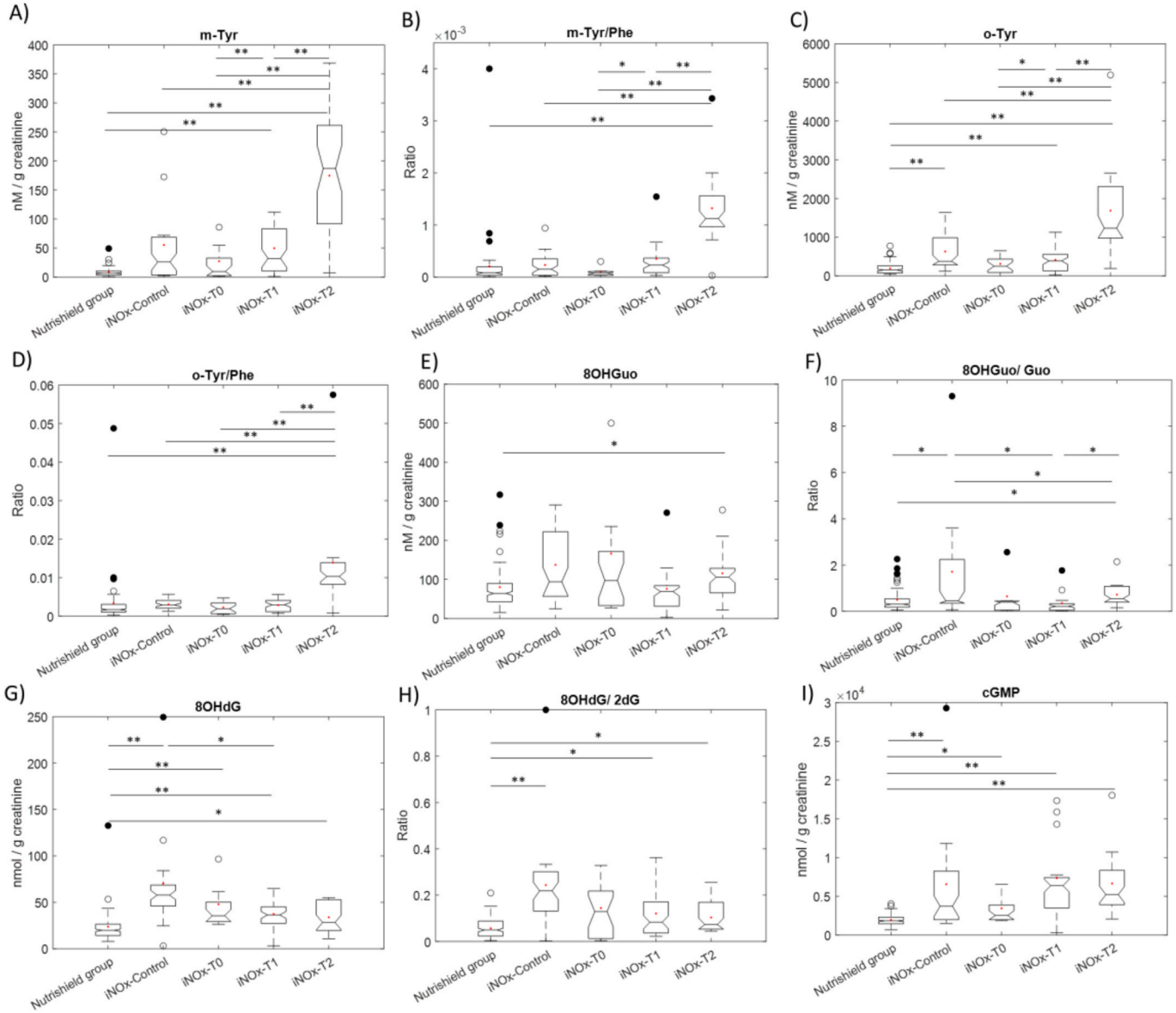


**Fig. 3.** Levels of oxidative/nitrosative stress-related biomarkers. Note: *(*p*-value <0.05) and **(*p*-value <0.001), Wilcoxon rank sum test.

has been observed in infants in an earlier study [2], suggesting that the effects of NO on lungs may be mediated through cGMP signaling. In this study we did not observe this effect of correlation between iNO and cGMP. Nevertheless, as depicted in Fig. 4, a significant (Pearson, *p*-values <0.001 for all biomarkers, except $3NO_2$-Tyr/*p*-Tyr with a *p*-value <0.05) positive linear relationship between the constant dose of iNO (ppm) normalized by weight (g) and time of NO exposure (days) with levels of oxidative/nitrosative stress-related biomarkers (m-Tyr, o-Tyr, m-Tyr/Phe, o-Tyr/Phe, and $3NO_2$-Tyr) was found in samples collected from the iNOx study obtaining r values between 0.4 and 0.6. These results suggest that dose/time of iNO exposure has a dependent relationship on generated oxidative/nitrosative biomarker levels.

In other studies, the use of iNO in premature infants has been evaluated. Sekar et al. found that iNO is feasible and reduces the need for oxygen during the resuscitation of premature infants [3] and Ballard et al. reported no significant differences between a control group and treated infants for 3-$NO_2$-Tyr and protein carbonylation in plasma samples [42]. Nevertheless, others studies suggest that conventional support and optimization of respiratory care should be prioritized [43]. Additionally, Dixon et al. [5] indicated that iNO has been associated with a significantly increased risk of cancer in children who were treated. While the use of iNO is still under scrutiny, the results obtained in this study revealed that oxidative and nitrosative stress biomarkers (m-Tyr, m-Tyr/Phe, o-Tyr, o-Tyr/Phe, and $3NO_2$-Tyr/*p*-Tyr) are present at significantly higher concentrations in infant urine after iNO exposure which suggests that iNO causes free radical-mediated damage to proteins. Further studies in infants not treated with iNO and similar severity need to be conducted to better elucidate the role of oxidative/nitrosative stress in severely ill infants.

This work is subjected to some limitations. The sample size of patients analyzed was small and patients were heterogeneous regarding to their clinical characteristics and underlying pathologies, limiting the generalizability of the findings, larger studies are needed to confirm and expand upon these findings. Table 2 highlights significant differences between the iNOx case group, which includes patients who are more severely ill, and the iNOx control group. These disparities limit the comparability of the two groups with respect to the effects of iNO treatment. Despite those between-group differences, we want to highlight that the longitudinal evaluation of subjects treated with iNO supported that oxidative/nitrosative stress biomarkers increased when comparing samples collected before and after the treatment within the iNOx case group. Furthermore, we acknowledge that the current study does not include follow-up data to correlate biomarkers with clinical outcomes. However, we are currently conducting evaluations of neurodevelopment at 24 months of age with the aim of enhancing our understanding of the relationship between oxidative/nitrosative stress biomarkers and clinical outcomes. In addition, some included biomarkers such as $8NO_2$-Gua and $8NO_2$-Guo were not readily detected in samples, limiting information available to assess nitrosative stress. The long-term storage stability of the metabolites in urine has not been evaluated in this study and should be considered in future research.

## 4. Conclusion

This study presents a new, non-invasive tool to measure the impact of oxidative/nitrosative stress in critically ill infants. Infant urine in the context of iNO treatment of PPHN has proven to be a versatile biofluid that provides a window into the body's internal processes without the need for invasive procedures or provoking discomfort to individuals. Infants from the iNOx and Nutrishield studies showed distinct patterns suggesting that even controls from iNO are different to the Nutrishield group. This also suggests the importance of evaluating patients' basal levels for assessing the impact of treatment on their redox status. We would like to highlight the increase of oxidative stress-related biomarkers (i.e., *m*-Tyr, *m*-Tyr/Phe, *o*-Tyr and *o*-Tyr/Phe) during iNO treatment, as well as the clear correlation between those biomarkers, in addition to $3NO_2$-Tyr/*p*-Tyr with iNO exposure. To the best of our knowledge, this is the first study evidencing oxidative/nitrosative stress in the context of iNO treatment for PPHN. These promising results evidence oxidative damage to proteins through the detection of elevated concentrations of specific biomarkers, suggesting the need for close attention to possible side-effects of iNO therapy. In summary, the results of this pilot study suggest that iNO may induce oxidative/nitrosative damage to proteins. The presented results provide new insights related

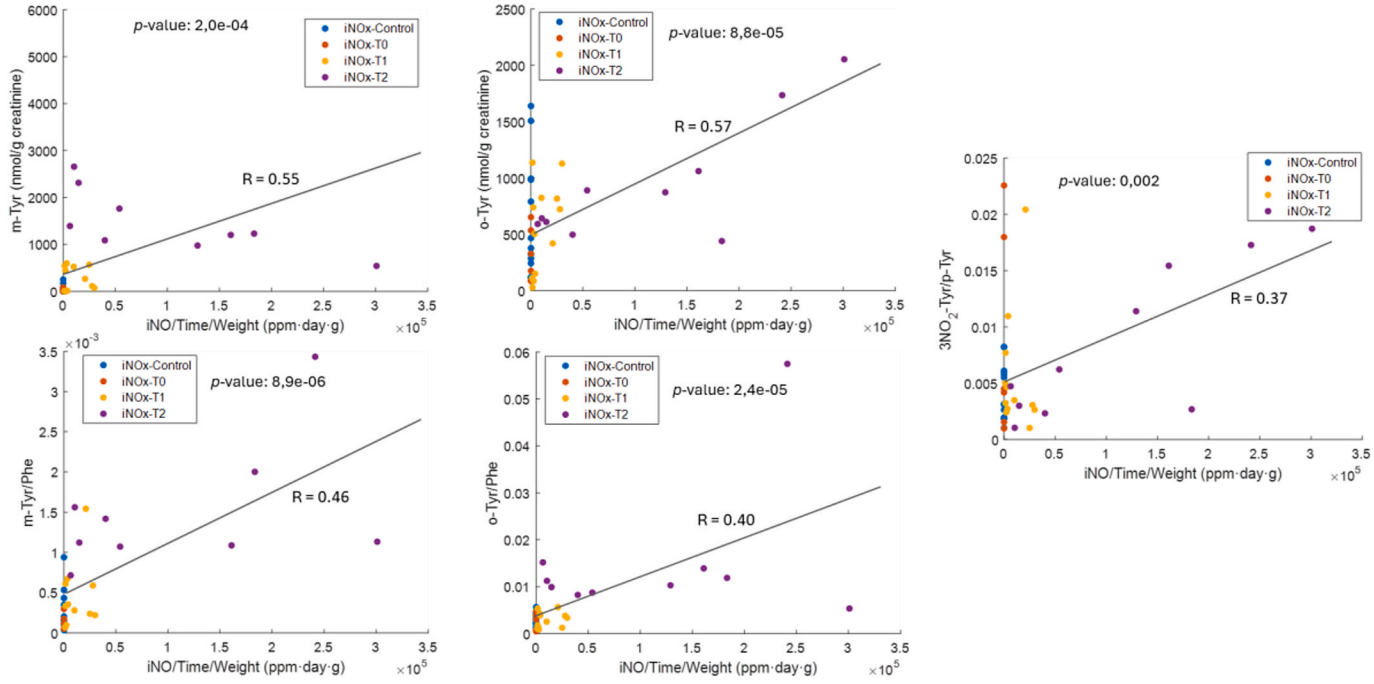


**Fig. 4.** Pearson linear relationship evaluation between *m*-Tyr, *o*-Tyr, *m*-Tyr/Phe, *o*-Tyr/Phe, and $3NO_2$-Tyr/*p*-Tyr biomarkers and iNO dose (ppm) normalized by time of exposure (days) and infant weight (g).

to the role of pulmonary vasodilation with iNO associated with ROS/RNS in newborn health. A follow-up evaluation of exposed neonates in the medium and long term is warranted to confirm the relevance of the observed early changes on the molecular level.

## CRediT authorship contribution statement

**Abel Albiach-Delgado:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alejandro Pinilla-González:** Resources, Methodology. **Mari Merce Cascant-Vilaplana:** Supervision, Methodology, Formal analysis, Data curation. **Álvaro Solaz-García:** Resources, Methodology. **Laura Torrejón-Rodríguez:** Methodology. **Inmaculada Lara-Cantón:** Methodology. **Anna Parra-Llorca:** Resources, Methodology, Data curation. **María Cernada:** Resources, Methodology. **María Gormaz:** Resources, Methodology. **África Pertierra:** Resources, Methodology, Data curation. **Caridad Tapia:** Resources, Methodology, Data curation. **Martin Iriondo:** Resources, Methodology, Data curation. **Marta Aguar:** Supervision, Resources, Methodology, Investigation, Data curation. **Julia Kuligowski:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Máximo Vento:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Julia Kuligowski reports financial support was provided by Carlos III Health Institute. Abel Albiach-Delgado reports financial support was provided by Carlos III Health Institute. Aljandro Pinilla-González reports financial support was provided by Carlos III Health Institute. Inmaculada Lara Cantón reports financial support was provided by Carlos III Health Institute. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2025.01.020.

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